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Zymosan Activation of TLRs: Stimulation of Innate Immunity and Nitric Oxide Production

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Abstract:

Chronic inflammation can result in pathophysiological changes to numerous organs of the body such as heart tissue (atherosclerosis) and damage to bone. Sources of inflammation can include autoimmune disease, cancer, and chronic infections such as those triggered by HIV or *Mycobacterium tuberculosis*. Most of the damage associated with chronic inflammation can be associated with chemical mediators, cytokines, given off by cells of the innate immune system. One measure of an active innate immune system can be assessed by quantifying nitric oxide (NO) production by bone marrow (BM) cells.

The purpose of these experiments is to determine if zymosan, a known inflammatory molecule associated with yeast cell wall, could stimulate cells of the innate immune response to produce cytokines capable of inducing NO production by BM cells. In these experiments mouse spleen cells were used as a source of innate immune cells and BM cells as the NO producing cell. In vitro, SpC and BM were cultured with yeast and NO production measured by the Greiss assay. In vivo mice were injected with yeast in the footpad and 48 hours later BM was removed and cultured and tested for NO production.

In vitro and in vivo experiments show that yeast could stimulate cells of the innate immune system to produce cytokines that could activate NO production. The levels NO produced were low but significant. The results show that yeast can activate cells of the innate immune system and potentially be a source of chronic inflammation.

Introduction:

Recognition of pathogens by cells of the immune system exists in two distinct immunological pathways. The host organism responds to a pathogen through a nonspecific innate immune response, followed by a specific adaptive response. The innate immune response relies on pattern recognition molecules that respond to a wide range of stimuli. The goal of this experiment is to observe the role a species of yeast, *Sarccharomyces cerevisiae*, plays in the activation of the innate immune system. Yeast are small, single cell organisms that play a role in the production of foods such as baked goods and beer. However yeast can also act as pathogens inducing tissue damage and disease. Yeast infections will stimulate cells of the innate immune system via pathogen-associated molecular pattern (PAMP)s found on their surface. Zymosan, found of the surface of yeast is the primary PAMP responsible for immune activation. This PAMP stimulates the toll like receptors (TLRs) within the innate immune system to trigger a response. Zymosan acts by binding to TLR-2 and TLR-6. TLR-2 plays a fundamental role in pathogen recognition and the activation of the innate immune response by the mediating the production of cytokines. It most commonly mediates a cytokine response to Gram positive bacteria and yeasts. TLR-6 functionally interacts with TLR-2 to mediate a cellular response to bacterial lipoproteins (Osamu, 2001). Once TLRs are activated, a number of possible cytokines can be released these include IL-8, IL-6, TNFα, and IL-12. Cytokines can travel throughout the body to activate physiological responses.

When the innate immune system is triggered, the cytokines that are produced will travel via the blood to effect many organ systems. Cytokines will stimulate the bone marrow to perform a number of functions, such as increasing the production of leukocytes that will enter circulation and

travel to sites of the inflammation. Initial cytokines will also stimulate additional responses from both BM and circulating leukocytes. One such response is the production of nitric oxide (NO) (Bogdan, 2001).

Nitric oxide is produced by macrophages, mature neutrophils, dendritic cells, osteoclasts and immature neutrophils. NO production is the result of the nitric oxide synthase. There are three forms of the NO synthase neuronal, endothelial and immune (Bogdan, 2001). Immune, also called inducible NO synthase (iNOS) is the form of the enzyme that is important for immunological responses. NO produced by cells of the immune system is important for destruction of pathogens, immune regulation and bone formation. While low levels of NO appear to promote bone formation high levels induce damage and potential osteoporosis (Hamilton, 2013). Osteoporosis is a common symptom of patients with chronic inflammation. NO produced by macrophages and pre-neutrophils are also associated with synovial fluid of patients with rheumatoid arthritis (Farrell, 1992). Inflammation caused by the autoimmune disease, recruits cells from the BM that upon arrival will be stimulated, amplifying the inflammatory response. In this environment NO is produced that will cause additional damage to the joint.

As implied transcription and translation of iNOS is induced by triggering cell surface receptors that will trigger the downstream events that will lead to enzyme synthesis. The iNOS promoter binds transcription factors involved in the activation of other genes by different cytokines. Transcriptional control of the promoter is fulfilled by a number of cytokines, such as the inducer role of IFN-γ in combination with LPS or another secondary signal (Kroncke, 1998). During transcription IFN-γ acts as an inducer, however, once transcription has occurred IFN-γ can act to stabilize the mRNA. The protein NF-kappa b acts to control transcription of iNOS (Xie and Nathan, 1994). Nitric oxide is produced by the bone marrow of the host organism when the innate immune response is triggered. A measure of this nitric oxide release by the bone marrow will indicate the amount of stimulation the innate immune system is undergoing in response to pathogens, such as zymosan.

The purpose of this paper is to determine if zymosan can act to stimulate NO production by BM cells in vivo and in vitro.

Materials and Methods:

Murine Organ Harvest

Syngenic C57Bl/6 female mice (Jackson Labs, Bar Harbor Main), 6 months of age were used in all experiments. We acquired in the experiment to ensure that the results do not deviate due to a specific specimen's immune system. Animal studies were approved by The University of Akron's Animal Care and Use Committtee. The mouse was sacrificed, using aseptic technique the spleen, tibias, and fibulas were harvested. The bones were cleaned to remove any remaining connective tissue and a scalpel was used to trim the edges and expose the bone marrow. Once removed the organs were placed in separate sterile petri dishes containing basal essential media (BEM) (Sigma, St Louis Mo.).

Singe Cell Suspension-Spleen

Under sterile conditions a single cell suspension of spleen cells was created. Excess fat present on the spleen was removed using a scalpel. Then the plunger of a 5cc syringe was used

to press the spleen through a 100 mesh stainless steel wire mesh. This released the spleen cells into the BEM solution. Once the cells were suspended in the BEM, they were transferred to a 15ml sterile centrifuge tube using a pasture pipet. The petri dish was then rinsed with fresh BEM. The test tube was then centrifuged for 7 minutes, 8°C, at 450 X g to form a pellet.

Single Cell Suspension-Bone Marrow

A single cell suspension of bone marrow was created by flushing the removed tibias and fibulas with BEM using a 22-gauge needle and syringe. The syringe was filled with BEM, and the needle was pressed into the cavity containing the bone marrow. The cells were collected and placed in to a centrifuge tube and treated as above.

Washing Cells and Red Blood Cell Lysis

After centrifugation the media was poured off and approximately 3 to 5 mL of TRIS-ammonia chloride buffer was added to the test tube, and the pellets were re-suspended. Tubes were incubated for five minutes to lyse red blood cells. After five minutes, the test tubes were filled to approximately 12 mL with fresh BEM and centrifuged. The supernatants were then poured off and the pellet was re-suspended in 4 mL of BEM. The test tube was filled with BEM and centrifuged. This acted as a wash cycle to ensure all of the TRIS buffer was removed. This wash cycle was repeated for the spleen cells. The pellet was then suspended in fresh tissue culture media.

After the second wash BM cells were placed in 7ml of tissue culture media (TCM) and placed into a 25cm^2 tissue culture flask (Costar) and incubated for 1hr in a humidified 6.0% CO_2 incubator at 37°C. This removes mature macrophages from the cell suspension, which are capable of both inducing and inhibiting nitric oxide production. After the incubation, the bone marrow cells were replaced in a test tube, filled with fresh TCM, and centrifuged.

Solution Protocols

Fresh TCM: 91.0 mL of manufacturer standard RPMI, 1.0 mL of 10 mM HEPES, 1.0 mL of $5x10^{-5}$ M 2-Mercaptoethanol, 1.0 mL of 200 mM glutamine, 1.0 mL of penicillin streptomycin (100 g streptomycin 100 units penicillin/mL), and 5.0 mL of 5% fetal calf serum. These chemicals were combined within the hood to avoid contamination, then filter sterilized through a 0.2um filter (Costar, Corning NY).

Yeast Solutions: The stock yeast solution was created using Red Star baking yeast. Exactly 22 mg of yeast was dissolved in 22 mL of PBS to form a 1 mg/mL solution. The solution was sterilized by autoclaving and then stored at 4℃. This stock solution was then used in a serial dilution to form the following solutions: 10 µg/mL, 1.0 µg/mL, 0.1 µg/mL, and 0.01 µg/mL. These concentrations of yeast (10, 1.0, 0.1, and 0.01 µg/mL) were chosen based upon a range determined by previous literature.

Cell Counts

To determine cell counts the cells were diluted in the viability stain trypan blue. The solution was loaded into a hemocytometer and counted. Once a count was achieved, the number of cells per milliliter was calculated using the following calculation:

Number of cells counted x dilution value x 10^4,hemocytometer volume = # cells per milliliter

Once the cells per milliliter was calculated, the cells were diluted to the desired concentration in TCM.

In vivo Mouse Experiment

The in vivo experiment was conducted using two mice. Mice were injected with zymosan 20ul of 1mg/ml into the footpad of both hind limbs. After 48 hours, the mice were sacrificed and their tibias and fibulas were removed.

Spleen Cell Supernatant Cultures

The wells of a sterile 96 well plate were added mixtures of TCM, 2.5X10^5 spleen cells/well and three different concentrations of yeast. A negative control, containing only spleen cells and media, and a positive control, containing spleen cells in combination with alpha galactose ceramide (α Gal-Cer) (DiagnoCine, Hackensack, NJ) (10 ng/mL) and media, were used as positive and negative controls respectively. Three different concentrations of yeast were combined with spleen cells: 10 µg/mL, 1 µg/mL, and 0.1 µg/mL. The α Gal-Cer stimulates NK T-cells, which makes it a prime candidate for a positive control that stimulates action in the innate immune cells (Kitamura, 1999). All wells contained a final volume of 250ul. The plate was incubated for 48 hours for cells of the innate immune system to respond with the production of cytokines. The supernatants were harvested and centrifuged at 2000 RPM for 12 minutes to remove spleen cells. After centrifugation the supernatants (sup) were separated from the cell pellet and placed in fresh test tubes. These supernatants were kept on ice until they were added to cultures of bone marrow cells.

Nitric Oxide Production

Cell cultures were carried out in 96 well flat bottom plates in a final volume of 250 µL. Each set of experimental cultures were run in triplicate. The different biological reagents used for plating included LPS (Sigma) (3.3 µg/mL), IFN-γ (Prospect, Revolt Israel) (25 units/mL or 5 units/mL), yeast of differing concentrations, cell supernatants, and cell suspensions. The well was adjusted to a final volume of 250ul using TCM. To determine the amount of NO produced in the culture 50ul of culture supernatant was removed at the time indicated in the figures and mixed with Greiss reagent. Cultures containing BM cells and stimulating agents were assayed at 48 and 72 hours, while cultures containing BM, Spc and yeast were assayed at 96 hours.

NO assay

NO is a gas that is rapidly reduced to nitrate in culture. A standard curve was generated by dissolving nitrate into 100, 50, 25, 12.5 and 6.25 uM concentrations. To 50ul of nitrate was added 50 ul of Greiss reagent and incubated for 5 minutes and then the absorbance determined at 450nm (Sun, 2003). The standard curve was used via a regression analysis to determine NO concentration per culture supernatant of nitrate.

Nitric Oxide Readings

After the plates were incubated for periods ranging between 48 and 96 hours, they were sampled for nitric oxide production. A total of 50 µL of each sample was placed into a clean well plate inside a Laminar flow hood. Exactly 50 µL of Greiss reagent was then added to the samples. After a 5 minute period, the well plate was placed within the spectrophotometer and the values for nitric oxide were read. These values were compared to the standard curve to determine the amount of nitric oxide produced by each sample using a regression analysis.

Statistics

To determine if a significant difference in NO production occurred a paired T test was performed.

Results:

The goal of the first experiment was to determine the effects of yeast on NO production for BM and SPC alone or in the presence of stimulating agents, such as IFN–γ and LPS, singularly or in combination. Figure 1 shows the results of the preliminary test of spleen cells in combination with bone marrow cells. As shown, the addition of yeast to the cultures increased NO production in a dose dependent manner. This experiment also included wells with 0.01 µg/mL yeast solution. This portion of experimental wells indicated an abnormally high concentration of NO production compared to both the Y=0.1 µg/mL and the control wells containing no yeast, suggesting some form of error.

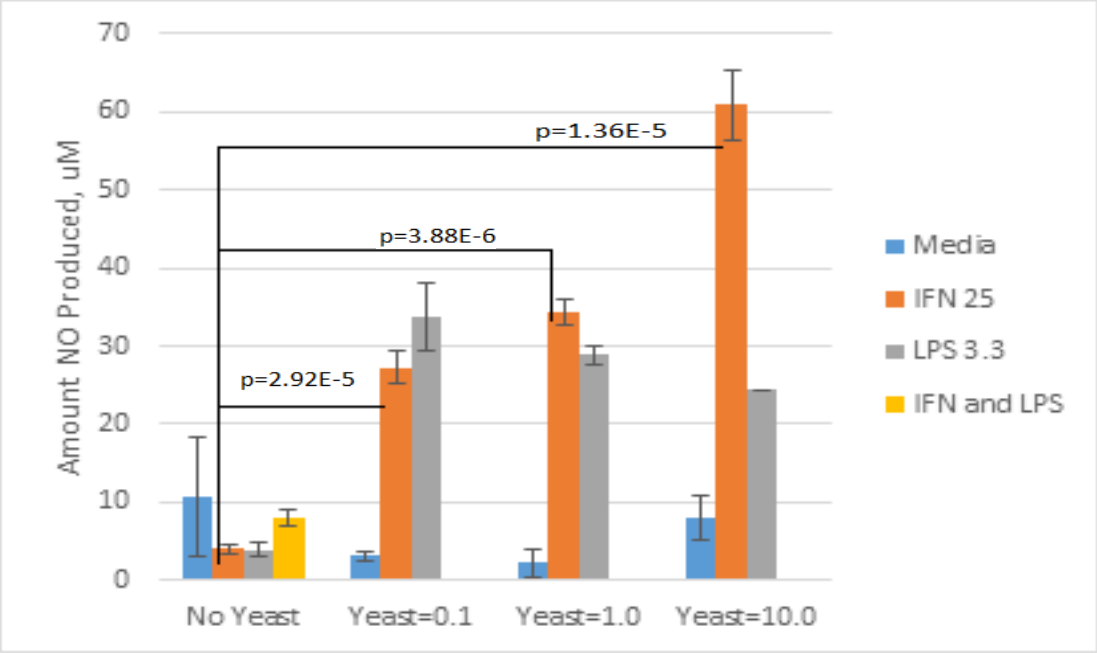
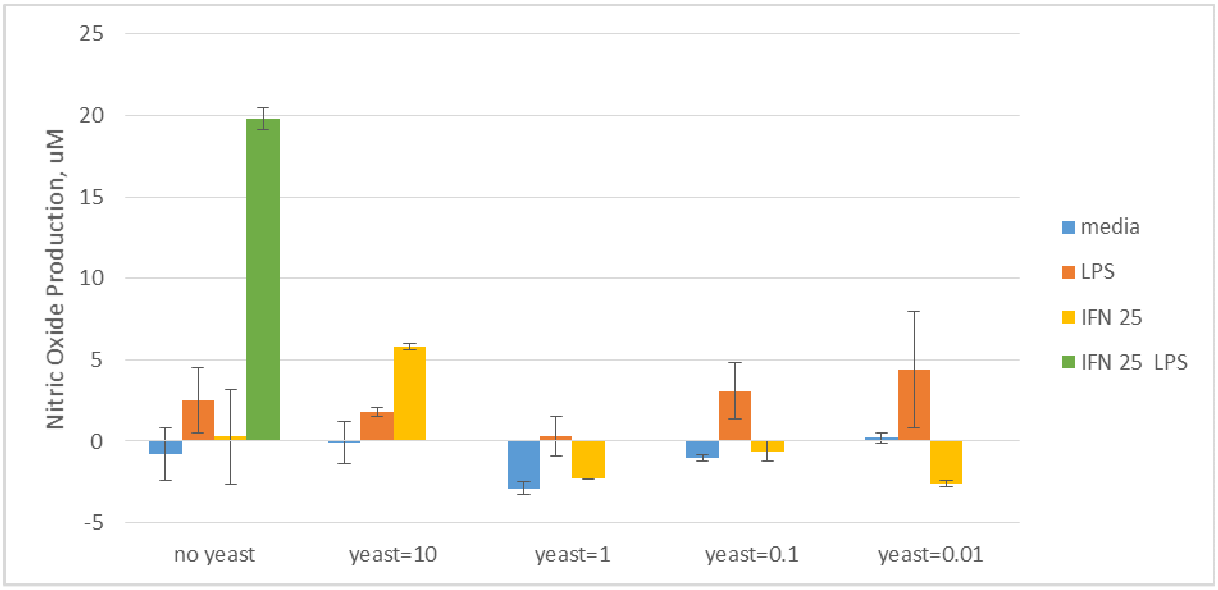


Figure 1: Nitric oxide production by cultures of BM and SPC incubated with *S. cerevisiae* with or without LPS or IFN-γ. Cultures containing $2.5x10^5$ cells/well spleen cells and $4.0x10^5$ cells/well BM were incubated with LPS (3.3 µg/mL) and IFN-γ (25 units/mL) with the indicated concentrations of yeast. Cultures were incubated for 72 hours and 96 hours, then the supernatants were tested for nitric oxide content.

As seen the largest increase in NO production came from cultures containing yeast and IFN–γ, suggesting that the yeast acted to stimulate cells of the innate immune system to produce a cytokine that acts synergistically with IFN–γ. The results in figure 1 do not indicate if the cells of the innate immune system responding to yeast is found in the SpC or BM cell population. Results in other systems suggest that the spleen is contributing the cells of the innate immune system that are stimulated by the yeast. The next experiment was performed to see if yeast could enhance NO production by BM cells in cultures without SpC.

The second experiment shows the effect that yeast has on bone marrow cells alone. The



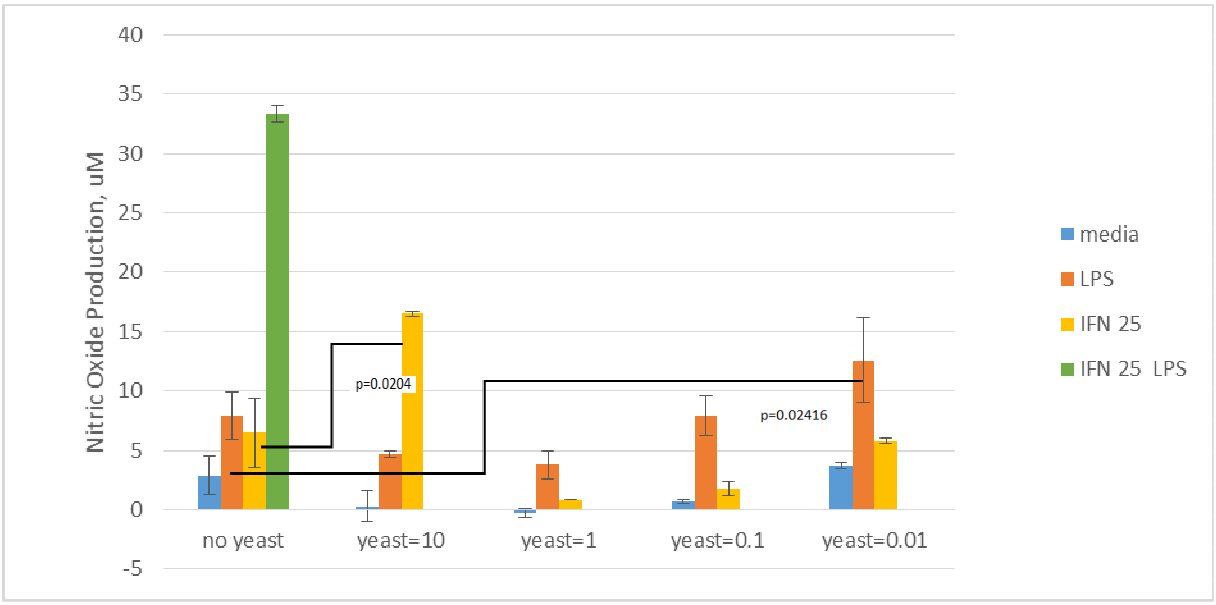


Figure 2: Nitric oxide with *S. cerevisiae* Cultures containing 3.5 x 10^5 BM cells were incubated with LPS (3.3 µg/mL) and IFN-γ with the indicated concentrations of yeast. Cultures were incubated for 48 (top graph) and 72 hours, then the supernatants were tested for nitric oxide content.

experiment was run using two concentrations of IFN–γ, 5 units/mL and 25 unit/mL, however the results indicated no significant difference when using the differing concentrations. IFN–γ 5 and IFN–γ 25 were chosen based upon their use in non-related experiments by Dr. Holda. After 48 hours of culture, the nitric oxide production was measured using the Greiss assay. Compared to the controls containing no yeast, the experimental wells did not produce any significant amounts of NO, though they indicated increased production at the 10 µg/mL yeast wells (Figure 2). The cells were returned to the incubator and retested for nitric oxide content at 72 hours. There was a low but significant result in cultures containing IFN–γ 25u the 10 µg/mL yeast wells (p=0.0204). Since the result was low, the experiment was repeated. The significant result was not reproducible. The result does suggest the intriguing possibility that there are a low number of cells in the BM that can respond to yeast. This possibility could be addressed in future experiments.

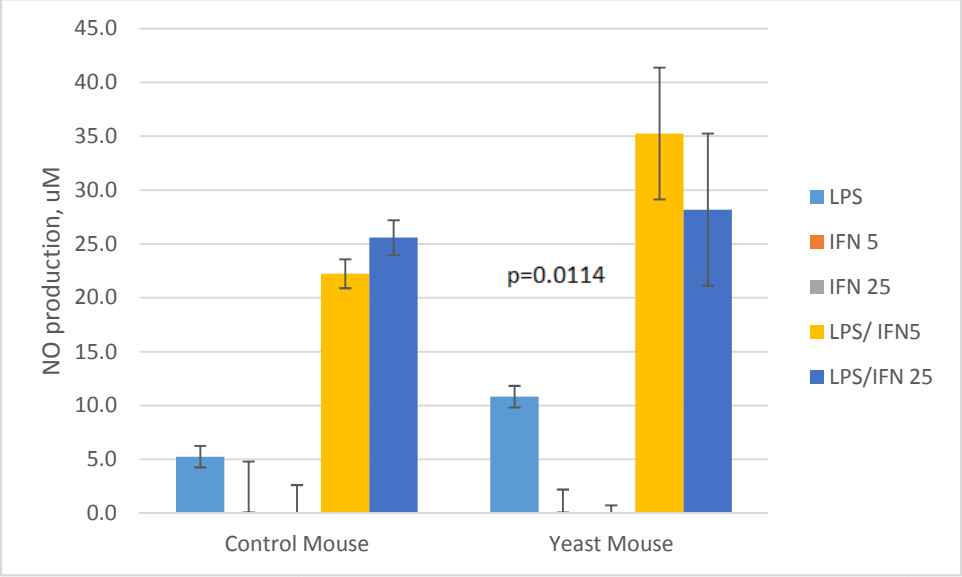


Figure 3: Injecting yeast into the foot pads of mice enhanced NO production by BM cells. Mice were injected in the hind foot pad with a yeast suspension. 48 hours later the BM cells were removed and cultured with the indicated reagent for 48 hours. Cultures containing the BM $4.0x10^6$ cells/well were incubated with LPS (3.3 µg/mL) and IFN-γ (25 units/mL or 5 units/mL) and either yeast (20 µg) or saline. Cultures were incubated for 72 hours, then the supernatants were tested for nitric oxide content. A significant change, p=0.0114, in NO production was seen in the experimental sample containing LPS and IFN-γ 5 units/mL.

The third experiment was to examine the effect of yeast in vivo on the innate immune system. A mouse was injected with 20ug of yeast in a yeast suspension in 20 ul in both foot pads, control mice received sterile PBS. After 48 hours, the mice were sacrificed and the bone marrow harvested and cultured with stimulating agents, seen in Figure 3. Culture supernatants were then tested for NO production after 48 and 72 hours of culture. Figure 3 shows the result of the 72hour culture. Cultures of BM cells from the mouse injected with yeast produced significantly more NO when cultured with LPS and 5 units of IFN–γ, p=0.0114.

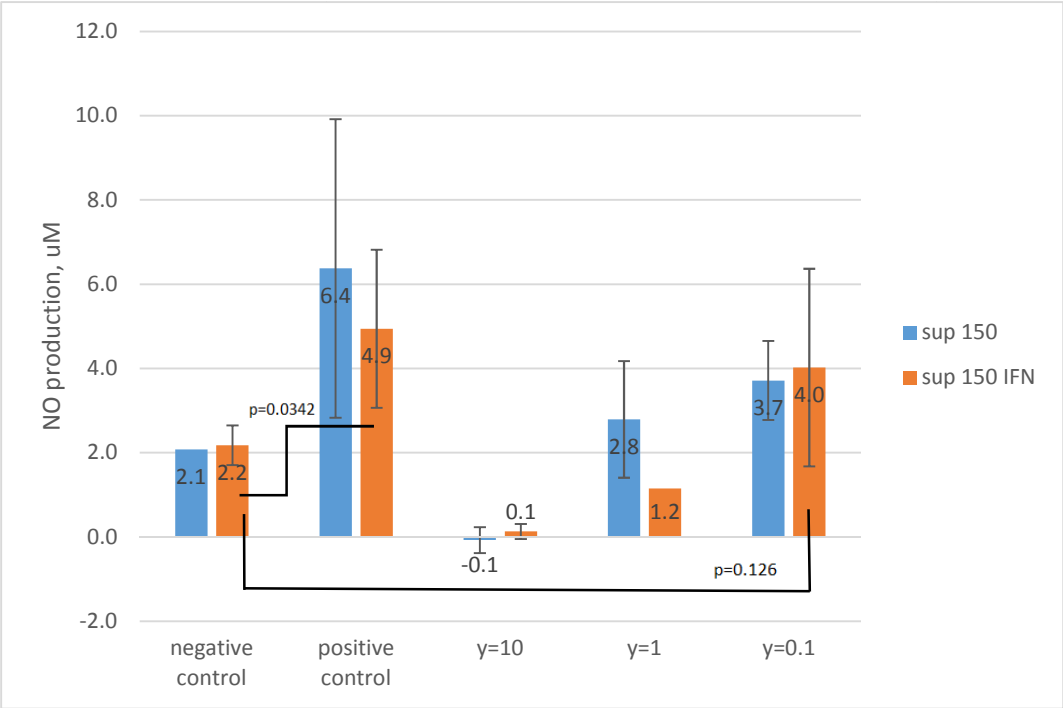


Figure 4: Nitric oxide production by cultures containing BM in combination with spleen cell supernatants and IFN (25 units/mL). The positive and negative control indicate a significant difference, p=0.0342, when supernatant was combined with IFN-25. Differences, though insignificant, were also detected when 150 µL of supernatant containing 0.1 µg/mL of yeast was combined with IFN-25. The supernatants of the SPC solution were combined with $4.0x10^6$ cells/mL of BM and incubated for 48 hours. The supernatants were then tested for nitric oxide content.

The final experiment was to see SpC could be stimulated by yeast, in vitro, to produce cytokines that could stimulate NO production by BM cells (Figure 4). SPC were incubated with yeast cell suspensions for 48 hours. After incubation, the supernatants were harvested and placed in cultures containing BM cells and IFN–γ. Culture supernatants from SpC incubated with αGal-Cer, were used as a positive control. BM cells were cultured with supernatant for an additional 48 hours then tested for NO production. Only one significant difference was present. It indicated that the positive control worked, based on the significant difference (p=0.0342) between the positive and negative controls containing 150 µL of supernatant and IFN–γ. These controls were then compared to the supernatants with yeast at 10 µg/mL, 1 µg/mL, and 0.1 µg/mL. Though none of the results indicate significant differences.

Discussion:

Nitric oxide is a gas, produced by cells of the body, which has numerous biological functions. NO has anti-microbial effects via its oxidizing activity on bacteria and viruses, and it has a role in molecular signaling and communication. NO associated with endothelia cells and neuronal cells is constitutively produced and important in vascular tonicity and neuronal development respectively (Melikian, 2009). Cells of the immune system only produce NO after stimulation by cytokines and/or by pathogens containing specific, PAMPs. Immune NO, referred to as inducible NO, is produced by the enzyme inducible nitric oxide synthase (iNOS). Transcription of the iNOS gene requires induction of two separate transcription factors: NF-kB and an interferon stimulated response element (ISRE)(Morris, 2003). The NF-kB factor is released from the cytoplasm of the cell after stimulation by a various

cytokines such as TNF-alpha and IL-1beta. In addition, LPS an integral compound in Gram negative bacteria is a very potent activator of NF-kB. Cells of the innate immune system are an excellent source of the cytokines that stimulate release of NF-kB. Stimulation of cells with interferon induces the transcription and translation of the ISRE factor. ISRE then binds to the promoter of the iNOS gene. Once the NF-kB factor binds with ISRE, the iNOS gene is transcribed. The purpose of these experiments is to determine if yeast particles can activate cells of the innate immune system to produce the cytokines that can activate the NF-kB pathway and NO synthesis.

The cell wall of yeast molecules contain the PAMP zymosan. Zymosan is known to bind to TLR-2 and TLR-6, receptors on the surface of the innate immune system and induce cytokine production (Paul, 2008). In the first experiment I wanted to determine if yeast could stimulate cells of the innate immune systems to produce cytokines that stimulate NO production. Yeast was added to cultures containing SpC, a source of innate immune cells, and BM. The BM cells contain the NO producing cells that interact with the cytokines produced by the SpC cells. Cultures were created with or without additional stimulating agents. This experiment showed that yeast could stimulate the production of cytokines that acted synergistically with IFN-γ to stimulate NO production. In the next experiments I wanted to determine if the SpC were necessary for NO production, or if cells of the low concentration of innate immune cells found in BM could be a source of cytokines. Results showed that a weak but significant amount of NO was produced from cultures containing only BM and yeast, but this significant result was not reproducible. One interpretation could be that while cells of the innate immune system are found in the BM their numbers are low and resulting in very low cytokine production.

To further examine the role of the innate immune cells, yeast was injected into the footpads of mice. After 48 hours, BM cells were removed and tested for NO production. Previous experiments using the adjuvant complete Freunds and the NK-T stimulating agent α-galactose cerimide resulted in an enhanced ability of BM to produce NO. It has been reported that injection of stimulating agents in the footpad can result in cytokine production within 17 hours that can act in an endocrine fashion on the BM (Vitoriano-Souza, 2012). Results from this experiment support the role of the innate immune system in activating NO producing cells.

Finally I wanted to determine if cell supernatants from SpC cultured with yeast could stimulate NO production when mixed with BM cells and IFN-γ. Spc were cultured with yeast (10, 1, or 0.1ug/ml), a-Gal Cer (positive control) or just media alone (negative control). NO production in this experiment was low, making the results difficult to interpret. BM cells incubated with media from SpC incubated with α-Gal Cer did produce a significant amount of NO when incubated with 150ul of conditioned media compared to the control (figure 4). The significant difference did not exist when BM cells were incubated with only 50 µL of supernatant, indicating that the cytokine(s) produced dilute out quickly. While media from SpC incubated with 0.1 ug/ml of yeast produce levels of NO above the control, they were not significant. The large standard error and low concentrations of NO produced, are the main reasons for the insignificant result. This indicates that with slight protocol modifications, a supernatant could be generated that would stimulate NO production in BM cells.

These results support the hypothesis that yeast cells can stimulate cells of the innate immune system to secrete cytokines which enhance NO production. In vivo experiments demonstrate that within 48 hours of injecting the yeast, cytokines are produced that act on the NO producing cells of the BM. This stimulation primes the BM cells for subsequent NO production upon stimulation. In vitro

experiments show that yeast has little effect on the BM, also suggesting that BM contains only a few cells responsive to the yeast particles. Significant amounts of NO were also produced when cultures contained all three ingredients: SpC, BM, and yeast. This provided the cultures with a source of innate immune cells, a source of NO producing cells, and the zymosan particles.

Yeast cell wall contains zymosan, a known pathogen associated molecular pattern (PAMP) molecule. Zymosan, has been shown to combine with TLR2 to stimulate production of cytokines such as IL-6 and IL-8. Both IL-8 and IL-6 activate the MAP kinase pathway and the NF-kB signaling pathway (Nomi, 2010). Other sources suggest that the cytokine IL-8 can also effect the ERK pathway, as well as the NF-kB pathway (Carta, 2013). Zymosan has been found to stimulate IL-8 production early in the innate immune response, at the point where neutrophils interact with mitogens (Au, 1994). IL-8 and IL-6 seem to be the top cytokine candidates in stimulating NO production. IL-6 is a pro-inflammatory cytokine secreted by both T-cells and macrophages, and IL-8 is an inflammatory cytokine produced by macrophages. Either may act as the secondary signal to stimulate the NF-kB receptor, then combine with IFN$-\gamma$ to activate the iNOS gene. It should be possible to identify the cytokines that zymosan produces by generating supernatants from SpC incubated with yeast. The initial experiment presented here did not give a significant result, however further experiments should be performed to attempt to optimize the experimental conditions.

Limitations:

This project was completed using common baking yeast, *Sarccharomyces cerevisiae*, which has one PAMP associated with it. There are other species of yeast containing different PAMPs that will stimulate TLRs other than TLR-2 and TLR-6. Pathogenic yeasts, such as the Cryptococcus genus and *Candidia albicans*, may have a different stimulating effect on the innate immune system. For example, *Candidia albicans* is an opportunistic pathogen that can invade the mucous membranes and cause significant damage when the immune system is compromised. This species of yeast has PAMPs such as mannan and O-linked mannosyl residues that activate TLR-4. TLR-4 will activate a different cytokine response that may produce a different amount of nitric oxide in the bone marrow, which indicates a difference in innate immunity stimulation.

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